AD_____

GRANT NO: DAMD17-94-J-4172

TITLE: Role of Raf-1 Signaling in Breast Cancer - Progression to Estrogen Independent Growth

PRINCIPAL INVESTIGATOR: Dorraya El-Ashry, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

REPORT DATE: 09/22/95

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	09/22/95	Annual 15 Jun 94 - 14 Jun 95

4. TITLE AND SUBTITLE
Growth Factor Regulation of Estrogen Receptor Function - A Pathway to Estrogen Independence

5. FUNDING NUMBERS
DAMD17-94-J-4172

6. AUTHOR(S)
Dorraya El-Ashry, Ph. D

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20057

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Material Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Breast cancer progression may be characterized by a switch from hormone-dependent to hormone-independent growth that involves several cellular alterations. Overexpression of many growth factor receptors has been shown to confer estrogen-independent growth on estrogen receptor positive breast cancer cells. The Raf-1 protooncogene is a key intermediate in the signaling pathway of many of these growth factor receptors. When a constitutively activated Raf was expressed in ER+ MCF-7 human breast cancer cells, the cells down-regulate expression of the transfected Raf. Constitutive Raf activity does allow for growth in the absence of estrogen, suggesting that activation of growth factor signaling pathways through Raf may confer selective advantage for growth under estrogen-deprived conditions. In addition, the high levels of Raf activity induce apoptosis in cells grown in the presence or absence of estrogen. This is a novel activity for Raf, and may occur due to the extremely high levels of Raf activity expressed in these transfectants. Therefore, it can be hypothesized that lower levels would mediate growth, while the higher levels result in additional, differential activation of other pathways.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Raf-1 Oncogene, Estrogen-Independent Growth, Apoptosis	31
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet ***optical scanning requirements***.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	**PR**	- Project
G	- Grant	**TA**	- Task
PE	- Program Element	**WU**	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

- **DOD** - See DoDD 5230.24, "Distribution Statements on Technical Documents."
- **DOE** - See authorities.
- **NASA** - See Handbook NHB 2200.2.
- **NTIS** - Leave blank.

Block 12b. Distribution Code.

- **DOD** - Leave blank.
- **DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
- **NASA** - Leave blank.
- **NTIS** - Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Doraya El-Ashry 9/22/95

PI - Signature | Date

Table of Contents

NOTIFICATION OF CHANGE IN SCOPE

On August 24, 1995, I requested approval from my Grants Officer, Ms. Patricia Shoop, for a change in scope to my Career Development Award entitled "Growth Factor Regulation of Estrogen Receptor Function--A Pathway to Estrogen Independence." The major hypothesis of this proposal was that growth factor signaling in human breast cancer cells would activate the estrogen receptor (ER) in the absence of estrogen, thus leading to estrogen independent growth, and that this represented an early step in the progression to estrogen-independence. The three specific aims were designed to study this question by analyzing the effects of growth factors on the activation of ER with the end-point of analysis being activation of its target DNA response element.

In the course of performing the experiments proposed in the Statement of Work for months 1-18, however, technical problems were encountered. Briefly, in repeating EGF treatment of the ERE-CAT stably-transfected MCE5 cell line, equivocal results were obtained making it difficult to quantitate the effects of EGF on ER activity. In addition, three attempts at stably transfecting the control MCNC4 cell line were unsuccessful. As a result, I felt a reassessment of how best to answer the major question proposed in this grant was necessary. I thought that examining the role of a key downstream effector of growth factor signaling in the progression to estrogen-independence might yield more productive results.

I hypothesized that the constitutive activation of the Raf-1 kinase in ER positive breast cancer cells (MCF-7) would result in the acquisition of estrogen-independent growth. And, should this occur, then these cells would make a useful model system to study the effects on ER activation. We therefore established several clones of MCF-7 cells stably expressing a truncated, constitutively active Raf and examined these clones for their ability to grow in the absence of estrogen. As hypothesized, the constitutive activation of Raf did result in estrogen-independent growth. In addition, two extremely interesting observations were made -- the constitutively active Raf also induced apoptosis in these cells, and cells grown long-term in the absence of estrogen lost ER.

In summary, the overall goal of the study has not changed but I feel that we now have a stronger model to study the effects of growth factor signaling in the growth of estrogen-dependent breast cancer cells. We can therefore examine the downstream effects of this constitutive Raf activity that lead to estrogen-independent growth, loss of ER, and the induction of apoptosis. Consequently, I would like to request a somewhat more general title: Role of Raf-1 Signaling in Breast Cancer--Progression to Estrogen Independent Growth.

On September 19, 1995, I was notified by Ms. Shoop that the requested changes in title and scope had been approved. The formal paperwork to officially designate these changes is in progress. She recommended that I write the annual progress report on the new line of investigation. As a result, I have written in this report a more extensive introduction than would normally be necessary, but I feel it is important to introduce this new subject material sufficiently.

INTRODUCTION

Breast cancer growth can be estrogen-dependent or -independent. Estrogen-dependent breast cancer, that is breast cancer that expresses functional estrogen receptors (ER) and requires estrogen for growth, has a better prognosis than estrogen-independent, ER negative breast cancer (1-4). This better prognosis is reflected in both a longer disease-free survival and overall survival, as well as, response to endocrine therapies such as the antiestrogen tamoxifen.

It is well established that estrogen treatment of ER+ breast cancer cells results in increased growth and the increased expression of several growth factors such as transforming growth factor-α (TGF-α) and insulin-like growth factor-I (IGF-I), as well as tyrosine kinase growth factor receptors such as the epidermal growth factor receptor (EGFR) [reviewed in (5,6). As a result, it has long been hypothesized that estrogen promotes cellular proliferation via the induction of specific growth factors and their cognate receptors thus setting up an autocrine loop. Upon progression to estrogen-independent growth, there is loss of ER expression and overexpression of certain growth factors/receptors, such as TGF-α and EGFR. Given this, it is also possible that signal transduction via growth factor tyrosine kinase receptors is a separate and alternate pathway and thus a mechanism for bypassing estrogen-mediated effects. Upregulation of these pathways may therefore be an early event in progression to the ER- phenotype. Many studies support this idea. Breast cancer cells overexpressing FGF-4, FGF-1, or heregulin become tumorigenic in nude mice in the absence of estrogen (7,8). Overexpression of EGFR or erbB-2 in breast cancer cells confers a growth advantage in estrogen-depleted media (8-10) and can increase tumorigenesis in nude mice in the absence of estrogen.

Most of the tyrosine kinase receptors like EGFR, erbB-2, and fibroblast growth factor receptor (FGFR) transduce their signals via the GTP-binding protein Ras [reviewed in (11-13)]. In the last couple of years, the pathways activated by Ras have been elucidated and many of the key intermediates have been identified. One of the major pathways initiated by the EGFR ligands is the mitogen activated protein kinase (MAP kinase or MAPK) phosphorylation cascade [reviewed in (14)]. Upon binding of ligand, the activated receptor interacts with and activates Ras, resulting in the subsequent activation of the Raf protooncogene. Raf-1, one of three Rafs [reviewed in (15,16)], is a serine/threonine kinase that can directly phosphorylate nuclear transcription factors like p53 (17). It also activates MAP kinase kinase (MAPKK or MEK), a dual specificity kinase that can phosphorylate both serine/threonine and tyrosine residues. MEK then phosphorylates MAPK on threonine and tyrosine, and the activated MAPK which is a serine/threonine kinase is able to phosphorylate several nuclear transcription factors including Myc, Elk, and Rsk (18-23). It is now known that not only can the activated Ras oncogene cause cellular transformation (24,25), but that activated v-Raf or a constitutively activated MAPKK can also result in transformation (26-29).

We were interested in studying the role of Raf in human breast cancer for several reasons. First, there are several studies indicating that oncogenic Ras transfected into ER+ MCF-7 cells renders them estrogen-independent. Dickson *et al* reported that oncogenic Ras in MCF-7s conferred estrogen-independent growth *in vitro* but not *in vivo*, and Sukumar *et al* obtained similar results (30,31). In studies by Kasid *et al* and Sommers *et al*, stable transfection of Ras into MCF-7 human breast cancer cells rendered them estrogen-independent in anchorage-dependent or -independent

growth and resulted in limited growth *in vivo*, suggesting that constitutive activation of the Ras pathway could either substitute for or bypass entirely the requirement of estrogen for the growth of these cells (32-34). While activating mutations of Ras are very rare in breast cancer, overexpression does occur in ~70% of breast cancer cases (35,36). This overexpression may have significant impact on the downstream effectors of Ras, such as Raf. Alternatively, the difficulty in obtaining estrogen-independent *in vivo* growth in the above studies might suggest that activation of signals downstream of Ras, such as Raf, may be limiting. A second reason to study Raf is that many other signaling systems such as protein kinase C (PKC) and protein kinase A (PKA) have been recently shown to activate or inactivate Raf respectively (37-39), suggesting that Raf may play a pivitol role in signal transduction by several different pathways known to be important in breast cancer.

In this study, we report that stable transfection of a constitutively active Raf kinase into MCF-7 cells results in estrogen-independent growth, but the cells remain responsive to estrogen. However, while the high Raf activity seems to allow for growth in the absence of estrogen, this high activity is not compatible with growth in the presence of estrogen and continued growth in estrogen-containing medium results in down-regulated expression of the transfected Raf. Perhaps most interesting is that high levels of Raf activity in these cells leads to apoptosis.

RESULTS

Characterization of Raf Transfectants for Δ-raf Expression and Kinase Activity. To begin to study the role of Raf in breast cancer cells, ER+ MCF-7 cells were stably transfected with a constitutively active Raf construct or a construct lacking the Raf cDNA. This construct consists of two cytomegalovirus (CMV) promoters where the first drives the expression of a hygromycin resistance gene and the second drives the expression of an amino terminal-truncated Raf (Figure 1). The deletion of the first 305 nucleotides of Raf results in a constitutively active kinase (26,28). After selection of single cell clones and their expansion, 15 hygromycin resistant clones were analyzed for expression of the transfected Raf (designated Δ-raf) by Western blotting. Of the 15, six were positive for expression of the ~34KDa truncated Raf. In addition, a pooled population of cells transfected with the vector alone was expanded and used as a control (Figure 2). An *in vitro* kinase assay was performed with these six clones to determine if the transfected Raf exhibited constitutive kinase activity. High levels of kinase activity, several-fold over that seen for endogenous Raf in the control pool, were obtained for each of the six clones (Table I).

These six clones and the vector control pool were maintained in media containing phenol red and 10% fetal bovine serum (FBS) or were switched to growth in media without phenol red and 10% charcoal stripped serum (CCS) to examine the growth in the absence of estrogen (Figure 2). At the point of this switch, the cells had been growing in FBS conditions for about 4 weeks. Over the first month in CCS, each of the clones appeared to be growing normally, whereas the control vector-transfected cells grew poorly, as expected of MCF-7 cells in the absence of estrogen. After 8 weeks in CCS, three clones were growing very well, as were their counterparts in FBS media. The other three clones had slowed considerably. After another 6 weeks the growth patterns of the clones in CCS were changing again; that is, the three clones that had been growing poorly now began to grow quite normally. Given these fluctuations in growth exhibited by the individual clones, we were

curious as to whether there had been alterations in the level of the transfected Raf expression over time in culture in FBS and CCS. We had previously seen a similar situation when EGFR or erbB-2 was stably overexpressed in MCF-7 cells, where the expression of the transfected EGFR or erbB-2 disappeared in the presence of estrogen (growth in FBS) but was stably high in the absence of estrogen (growth in CCS) (9,10).

Loss of Δ-raf Expression Occurs in FBS-Containing but not CCS-Containing Medium. Figure 3A shows Western blot analysis of Δ-raf expression in the six clones at the various time points of growth in FBS or CCS described above. Each of the six clones exhibited varying degrees of decreased expression of Δ-raf after 8 weeks in FBS and increased expression when grown in CCS. One very interesting clone, clone 35, exhibited an almost complete loss of expression in 8 weeks in FBS as well as in CCS. Initially, this clone grew very poorly in the absence of estrogen, yet upon resumption of normal growth after 14 weeks in CCS, it showed a dramatic increase in Δ-raf expression. These data suggest that Δ-raf expression confers a growth advantage in the absence of estrogen and that the loss in expression observed was not a result of plasmid loss, since expression could be increased again by shifting growth conditions. To confirm that plasmid loss was not occurring, southern analysis of DNA obtained from cells initially after thawing from freeze down (high levels of Δ-raf expression) and from cells after long-term growth in FBS (significantly decreased levels of Δ-raf) was performed. Even in clone 35, which has virtually no transfected Raf expression after months in FBS, the plasmid was still present in the same apparent copy number and with no evidence of rearrangement (data not shown).

Clones that had been growing for 14 weeks in CCS and were stably expressing high Δ-raf levels (ccs-lt) were switched back to growth in FBS and analyzed after every two passages for Δ-raf expression. Both clone 8 and clone 27 showed a decrease in Δ-raf expression after just two passages in FBS (Figure 3B). Further decreases occured with continued passage in FBS, a total of ~10-fold for clone 8c over 14 passages and ~6-fold for clone 27c over 6 passages. Continued growth in CCS for another 6 weeks (ccs-lt2) does not result in decreased Δ-raf expression. These data, along with the FBS data of the other clones, suggest both that high levels of constitutive Raf activity are incompatible with growth in the presence of estrogen, and that the increased Δ-raf levels seen in CCS are reversible.

We were next interested in determining the rate of Δ-raf loss in FBS. The clones were thawed again from the initial freeze-downs, and then monitored after every two passages of growth in FBS or CCS for Δ-raf expression. Shown in Figure 4 are three representative clones which exhibit different rates of loss of the transfected Raf, with clone 35 having the fastest and most complete loss. Clone 14 shows a small, but reproducible decrease in Δ-raf, ~1.5-fold. Clone 35 has a very rapid and significant reduction, ~6-fold by passage 8 and ~14-fold by passage 20. Clone 27 has a more moderate rate but still significant loss, ~3-fold by passage 8 and ~12-fold by passage 20. It is of interest to note that clone 35, which in the original experiments had problems growing in CCS, also had a significant decrease in Δ-raf by passage 4. Since the cells are passaged once a week (if growing in FBS), and since in the original experiments there was a lag time of about 4 weeks before switching to growth in CCS, it would appear that the reason for the initial poor growth in CCS was due to the fact that there had already been major reductions in Δ-raf levels. The cells resumed normal growth in CCS only after they had been able to upregulate Δ-raf levels again. In

confirmation of this, none of the clones exhibited significant growth problems in CCS when placed into CCS immediately upon thawing out. Furthermore, there was no decrease in Δ-raf expression in the initial growth in CCS as there had been in the original set of experiments (Figure 3A); as shown with clone 27, the levels actually increase with increasing passages in CCS, ~1.5-fold (Figure 4). Thus these data confirm that growth in FBS is incompatible with the expression of high levels of Raf kinase activity, but that these levels allow for growth in the absence of estrogen.

Estrogen Is Not Sufficient for Down-Regulation of Δ-raf. Because the main reagent, but not the only one, that is removed from serum upon charcoal stripping is estrogen, it seemed likely that it was the estrogen in FBS-containing medium that was inducing the loss in Δ-raf expression. To see if this was the case, cells growing long-term in CCS were switched to growth in CCS supplemented with 10^{-9} M 17β-estradiol. Again, the cells were analyzed by Western blotting every two passages to follow Δ-raf expression, and while there are reductions in Δ-raf levels, they are not as rapid nor to the same extent as those that occur in FBS, a maximum of about 2-fold for raf 35 at passage 4 (Figure 5). Whether longer passage in estrogen will result in the same total decreases observed in FBS or whether estrogen is only partially responsible for the decreases is not yet known.

Δ-raf Message Levels are Also Decreased in FBS. Northern analysis of the transfected Raf was performed to determine if the decreases observed in protein expression correlated with decreases in mRNA levels as well. RNA prepared from cells just after thawing (higher levels of Δ-raf protein expression) was compared to that from cells in long-term FBS (lower or no Δ-raf protein expression) for the transfected Raf mRNA and GAPDH. For each clone, the loss of Δ-raf protein expression correlates with a decrease in mRNA expression (Figure 6). In addition, RNA was prepared from clones growing in CCS for several passages. As with protein expression, these clones expressed higher levels of RNA for Δ-raf than their FBS early passage counterparts.

High Δ-raf Levels Confer the Capacity for Anchorage-Dependent and -Independent Growth. We next directly assessed the growth capabilities of these clones, both in FBS and in CCS. The clones were thawed from early freeze-downs so that they were expressing high levels of Δ-raf, and then they and the control pool were quick-stripped to remove estrogen before being plated in CCS. In anchorage-dependent growth assays, HCopool, raf14, raf27, and raf35 have similar doubling times in FBS: 28, 27, 24, and 27 hours, respectively (Figure 7A). Growth of HCopool in CCS, however plateaus at ~day 4, and has a doubling time of 172 hours over the course of the assay. The doublin times for raf 14 (41 hours), raf 27 (47 hours), and raf 35 (78 hours), while longer than those for growth in FBS, indicate the ability of the Δ-raf to allow for growth in the absence of estrogen. It should be noted that the Δ-raf expression levels of raf 35 in this experiment were greatly reduced compared to raf 14 and raf 27, corresponding to the increased doubling time displayed by raf 35 in CCS.

Clones were also assessed for growth in soft agar. As compared to the control cells, both raf 14 and 27 formed colonies in the absence of estrogen (CCS): ~300 colonies for raf 14 and ~500 colonies for raf 27 compared to ~50 for the control pool. Raf 14 and 27 were still responsive to estrogen in that they formed more colonies in CCS + E_2, and the antiestrogens tamoxifen and ICI had little or no effect on colony formation indicating the true absence of estrogen in the CCS (Figure 7B). To further demonstrate the effects of Δ-raf expression on the ability to form colonies in soft agar in the absence of estrogen, we also assayed early passage and late passage raf 27. Early passage raf 27

again was able to form colonies in CCS, ~300, whereas late passage raf 27 (no Δ-raf expression) was no longer capable of colony formation in CCS, ~50 colonies (Figure 7B). Colony formation in FBS, however, was very similar to both passage numbers, ~1000 for early passage and ~1450 for late passage.

High Δ-raf Induces Apoptosis. A striking feature of these clones when growing in CCS is their morphology. As shown in Figure 8A and B, there appear to be two distinct cell morphologies when the cells are grown on plastic. The adherent cells grow in spheroid clusters rising from the cell monolayer (Figure 8A). In addition, there are a large number of floating cells. Some of these floating cells represent the cell clusters detaching from the monolayer. The majority of floating cells, however, are rather large clear cells with the nucleus pushed over to one side (Figure 8B). The clones growing in FBS also exhibit these general characteristics but to a lesser extent. We were interested in determining what these cells represented, and so we assayed the clones for their apoptotic index using a cell death ELISA assay which is designed to detect histone-bound DNA fragments in the cytoplasm. All of the clones growing in CCS have high apoptotic indices, compared with the control pool growing either in FBS or stripped of estrogen (Figure 8C). The high apoptotic index seems to correlate with Δ-raf expression since in FBS, raf 35 with high levels of Δ-raf has a high apoptotic index but after long-term growth in FBS (no Δ-raf expression), these cells now resemble the control pool. When the floating cell populations were collected and assayed separately from the adherent cells, it was clear that these floating cells represent the apoptotic cells (Figure 8D). Estrogen added to cells growing in CCS for 1-2 weeks did not have a significant impact on the levels of apoptosis observed; specifically it did not appear that estrogen treatment for this length of time could reverse the apoptosis.

DISCUSSION

Given the importance of growth factor signaling systems in breast cancer, i.e. overexpression of EGFR or erbB-2 correlates with poor prognosis and overexpression of Ras in a high percentage of breast tumors, we were interested in examining a key intermediate in the propogation of these signaling pathways -- Raf-1 -- in breast cancer. One goal of this study was to determine the effects of constitutive Raf-1 kinase activity on the estrogen-dependent growth of breast cancer cells, specifically the ER+ MCF-7 human breast cancer cell line.

We therefore constructed a constutively active form of Raf by deleting the amino-terminal domain, thus leaving only the catalytic domain. This construct was stably transfected into ER+ MCF-7 cells and six, high expressing clones were selected for further analysis. During the course of this study, we observed that continued passage of the clones in the presence of estrogen (FBS and phenol red) resulted in a loss of expression of the transfected Raf. This loss is reflected at both the protein (Figures 2 and 3) and the message (Figure 5) levels, despite the continued presence of intact integrated plasmid sequences. We previously observed similar results when EGFR or c-erbB-2 was stably overexpressed in MCF-7 cells; that is growth of the transfected cells in estrogen-containing medium resulted in decreased expression of both protein and message but not loss of the plasmid (9,10). While the parent vectors used in these transfections were similar (Figure 1), when FGF-4

was expressed using these vectors, the same pattern of down-regulation in FBS and up-regulation in CCS was not observed (9). Additionally, several other factors including collagenase IV and chloramphenicol acetyl transferase have been expressed in these vectors with no apparent problems. It appears, therefore, that this pattern of down-regulation occurs only when molecules that result in hyper-activation of growth signaling pathways other than the estrogen pathway are over-expressed. It would seem, then, that the constitutive activation of these growth signaling pathways is incompatible with estrogen-induced growth of these cells and that the estrogen pathway dominates.

While growth of the transfectants in estrogen-containing medium was not compatible with high levels of constitutive Raf expression, these same high levels did confer a growth advantage to the cells in the absence of estrogen. All of the clones were able to grow in CCS medium but the control cells were not (Figure 7A). Not only did growth in the absence of estrogen not cause a down-regulation of the Δ-raf, but the level of expression actually increased over time in culture over that seen in the FBS cells just after thawing (Figure 3). These high levels of Δ-raf expression also allowed for colony formation in soft agar in the absence of estrogen (Figure 7B). Previous work by others using EGFR, c-erbB-2, and the activated Ras oncogene has shown similar results (8-10,30-34). Both EGFR and c-erbB-2 overexpression allow for estrogen-independent growth *in vitro*, but only one clone which had a consitutively activated c-erbB-2 was able to grow *in vivo* in the absence of estrogen(10). Activated Ras can also confer a growth advantage to cells in the absence of estrogen both in anchorage-dependent and -independent growth assays. However, only two studies which used a Ras construct under the control of multiple long terminal repeats observed in *vivo* tumor formation in the absence of estrogen (32-34). It is not surprising therefore, that Raf as a downstream effector of Ras activity would result in estrogen-independence when constitutively active. It remains to be determined whether the estrogen-independence we have observed with Raf *in vitro* will also occur *in vivo*.

Since estrogen is the main component in serum that is removed upon charcoal stripping, we added estrogen back to the cells growing in CCS to determine that it was estrogen in FBS which caused the down-regulation of Δ-raf. While some decrease in expression levels was observed when cells were passaged in CCS + E_2 (Figure 5), it was not to the same extent as that which occurred in FBS. Two possibilities for this exist. First, since the cells grow slower in CCS + E_2 than in FBS and since the rate of Δ-raf loss may be a reflection of the doubling time of the cells, it may take more passages in CCS + E_2 to see the same degree of down-regulation. Alternatively, it may be that estrogen alone is not responsible for the decreased expression and that it is estrogen acting in concert with some other factor which is also removed by stripping that causes the down-regulation. Both possibilities are under investigation.

The most interesting feature of the transfectants was their morphology. In contrast to control cells which grow in a flat monolayer and produce very few floating cells in FBS or CCS, the transfectants grow in CCS as spheroid clusters of cells rising out of the monolayer and produce a large number of floating cells that consist of these spheroids and a population of cells that appears as large, clear cells with the nucleus pushed to one side. These floating cells have a very high apoptotic index. It is not so surprising that the floating cells themselves are apoptotic, rather it is the extent to which these cells produce large numbers of floating cells that differs. Certain cell systems,

such as colon carcinoma cells, undergo apoptosis at a very low rate during normal culture of these cells (40,41). The apoptotic cells are floaters, and the number of floaters can be significantly increased by agents that specifically induce apoptosis in these cell types.

In MCF-7 cells, however, there is some disagreement on whether these cells can undergo apoptosis *in vitro*. It is well known that breast epithelial cells and breast cancer cells *in vivo* undergo apoptosis in response to estrogen withdrawal (42,43), but there has been an inability to measure this in cells growing *in vitro*, at least using the usual hallmarks of apoptosis: DNA laddering and morphology. With time-lapse microscopy providing more detailed visualization of cells, apoptosis has been observed in both MCF-7 and ZR-75-1 cells *in vitro* in response to estrogen withdrawal or treatment with the antiestrogen toremifene, although no DNA laddering was observed (44). Some investigators have used non-random DNA cleavage into large (several to several hundred kb) fragments as a measure of apoptosis (45) and found that by this criterium, estrogen-withdrawal resulted in cell detachment and DNA cleavage (46). The antiestrogen ICI 182,720 increased these parameters 2-fold, however the typical morphological alterations and DNA laddering of apoptosis were not observed. Using the new cell death ELISA assay, as we have used in our studies, investigators have been able to show that treatment of MCF-7 cells in vitro with VP-16, taxol, or tamoxifen does result in apoptosis (47,48).

Using this same assay, we show that the control pool exhibits virtually no apoptosis and that estrogen-withdrawal of these cells for up to 5 days does not increase the apoptotic index. The Δ-raf transfectants, however, have a high apoptotic index in CCS and in FBS, and this appears to be dependent on high Δ-raf levels since long-term passage in FBS which down-regulates Δ-raf also abrogates the apoptosis (Figure 8C). The fact that high levels of raf activity result in increased apoptosis in these cells provides a possible explanation for why the cells in FBS down-regulate Δ-raf expression. When other growth-signaling pathways are operative in the cell, a selection against a pathway that leads to apoptosis occurs. On the other hand, the cells cannot grow in the absence of estrogen without the constitutive Raf signaling, and therefore they upregulate its expression and subsequently undergo apoptosis. However, a balance between growth stimulation and apoptosis must exist because all of the cells do not die. Either enough growth stimulation occurs such that a threshold level of apoptosis cannot be surpassed or the induced apoptosis is likely to be a stochastic process in cells with high Raf activity.

Unlike the difficulty in demonstrating apoptosis in ER+ breast cancer cells lines, the MDA-MB-468 human breast cancer cell line which is ER- and overexpressess EGFR due to gene amplification (49), readily undergoes apoptosis as demonstrated by DNA laddering in response to EGF treatment (50). High doses of EGF cause growth inhibition and apoptosis in these cells suggesting that hyper-induction of growth factor signaling in these cells due to their EGFR overexpression may actually result in cell death as opposed to growth. It may be then, that our constitutive Raf transfectants undergo apoptosis via the same mechanisms and that the EGF induced apoptosis in MDA-MB-468s occurs through the Raf signaling pathway.

A major question arising from these observations is how does constutive activation of a growth pathway ultimately result in the opposite -- increased cell death. The focus of ongoing investigation is thus to determine what the effect of constitutive Raf activity on the expression and function of known potentiators of apoptosis, and what the downstream effectors of this constutive

Raf activity are that are responsible for the increased apoptosis.

REFERENCES

1. Knight WA, Livingston RB, Gregory EJ, McGuire WL. Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer. Cancer Res 1977;37(12):4669-71.

2. De Sombre ER, Thorpe SM, Rose C, Blough RR, Andersen KW, Rasmussen BB, King WJ. Prognostic usefulness of estrogen receptor immunocytochemical assays for human breast cancer. Cancer Res 1986;46(8 Suppl):4256s-64s.

3. Clark GM, McGuire WL. Steroid receptors and other prognostic factors in primary breast cancer. Semin Oncol 1988;15(2 Suppl 1):20-5.

4. McGuire WL, Tandon AK, Allred DC, Chamness GC, Clark GM. How to use prognostic factors in axillary node-negative breast cancer patients [see comments]. J Natl Cancer Inst 1990;82(12):1006-15.

5. Dickson RB, Lippman ME. Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. Endocr Rev 1987;8(1):29-43.

6. Lippman ME, Dickson RB. Mechanisms of normal and malignant breast epithelial growth regulation. J Steroid Biochem 1989;34(1-6):107-21.

7. McLeskey SW, Kurebayashi J, Honig SF, Zwiebel J, Lippman ME, Dickson RB, Kern FG. Fibroblast growth factor 4 transfection of mcf-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. Cancer Res 1993;53(9):2168-77.

8. Pietras RJ, Arboleda J, Reese DM, Wongvipat N, Pegram MD, Ramos L, Gorman CM, Parker MG, Sliwkowski MX, Slamon DJ. HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene 1995;10:2435-46.

9. Miller DL, El-Ashry D, Cheville AL, Liu Y, McLeskey SW, Kern FG. Emergence of mcf-7 cells overexpressing a transfected epidermal growth factor receptor (egfr) under estrogen-depleted conditions: evidence for a role of egfr in breast cancer growth and progression. Cell Growth Differ 1994;5(12):1263-74.

10. Liu Y, El-Ashry D, Chen D, Ding IYF, Kern FG. MCF-7 breast cancer cells overexpressing transfected *c-erb*B-2 have an *in vitro* growth advantage in estrogen-depleted conditions and reduced estrogen-dependence and tamoxifen-sensitivity *in vivo*. Breast Cancer Research and Treatment 1995;34:97-117.

11. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. Cell

1990;61(2):203-12.

12. Hunter T, Karin M. The regulation of transcription by phosphorylation. Cell 1992;70(3):375-87.

13. Fantl WJ, Johnson DE, Williams LT. Signalling by receptor tyrosine kinases. Annu Rev Biochem 1993;62:453-81.

14. Hill CS, Treisman R. Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell 1995;80(2):199-211.

15. Li P, Wood K, Mamon H, Haser W, Roberts T. Raf-1: a kinase currently without a cause but not lacking in effects. Cell 1991;64(3):479-82.

16. Magnuson NS, Beck T, Vahidi H, Hahn H, Smola U, Rapp UR. The raf-1 serine/threonine protein kinase. Semin Cancer Biol 1994;5(4):247-53.

17. Jamal S, Ziff EB. Raf phosphorylates p53 *in vitro* and potentiates p53-dependent transcriptional activation *in vivo*. Oncogene 1995;10:2095-101.

18. Siegfried Z, Ziff EB. Altered transcriptional activity of c-fos promoter plasmids in v-raf-transformed nih 3t3 cells. Mol Cell Biol 1990;10(11):6073-8.

19. Kolch W, Heidecker G, Lloyd P, Rapp UR. Raf-1 protein kinase is required for growth of induced nih/3t3 cells. Nature 1991;349(6308):426-8.

20. Alexandropoulos K, Qureshi SA, Bruder JT, Rapp U, Foster DA. The induction of egr-1 expression by v-fps is via a protein kinase c-independent intracellular signal that is sequentially dependent upon haras and raf-1. Cell Growth Differ 1992;3(10):731-7.

21. Blenis J. Signal transduction via the map kinases: proceed at your own rsk. Proc Natl Acad Sci U S A 1993;90(13):5889-92.

22. Thomas G. Map kinase by any other name smells just as sweet. Cell 1992;68(1):3-6.

23. Crews CM, Alessandrini A, Erikson RL. Erks: their fifteen minutes has arrived. Cell Growth Differ 1992;3(2):135-42.

24. Barbacid M. Ras genes. Annu Rev Biochem 1987;56:779-827.

25. Trahey M, Milley RJ, Cole GE, Innis M, Paterson H, Marshall CJ, Hall A, McCormick F. Biochemical and biological properties of the human n-ras p21 protein. Mol Cell Biol 1987;7(1):541-4.

26. Heidecker G, Huleihel M, Cleveland JL, Kolch W, Beck TW, Lloyd P, Pawson T, Rapp UR. Mutational activation of c-raf-1 and definition of the minimal transforming sequence. Mol Cell Biol 1990;10(6):2503-12.

27. Cleveland JL, Troppmair J, Packham G, Askew DS, Lloyd P, Gonzalez-Garcia M, Nunez G, Ihle JN, Rapp UR. V-raf suppresses apoptosis and promotes growth of interleukin-3-dependent myeloid cells. Oncogene 1994;9(8):2217-26.

28. Samuels ML, Weber MJ, Bishop JM, McMahon M. Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase. Mol Cell Biol 1993;13(10):6241-52.

29. Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande Woude GF, Ahn NG. Transformation of mammalian cells by constitutively active map kinase kinase. Science 1994;265(5174):966-70.

30. Dickson RB, Kasid A, Huff KK, Bates SE, Knabbe C, Bronzert D, Gelmann EP, Lippman ME. Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17 beta-estradiol or v-ha-ras oncogene. Proc Natl Acad Sci U S A 1987;84(3):837-41.

31. Sukumar S, Carney WP, Barbacid M. Independent molecular pathways in initiation and loss of hormone responsiveness of breast carcinomas. Science 1988;240(4851):524-6.

32. Kasid A, Lippman ME, Papageorge AG, Lowy DR, Gelmann EP. Transfection of v-rash dna into mcf-7 human breast cancer cells bypasses dependence on estrogen for tumorigenicity. Science 1985;228(4700):725-8.

33. Kasid A, Knabbe C, Lippman ME. Effect of v-rash oncogene transfection on estrogen-independent tumorigenicity of estrogen-dependent human breast cancer cells. Cancer Res 1987;47(21):5733-8.

34. Sommers CL, Papageorge A, Wilding G, Gelmann EP. Growth properties and tumorigenesis of mcf-7 cells transfected with isogenic mutants of rash. Cancer Res 1990;50(1):67-71.

35. Ohuchi N, Thor A, Page DL, Hand PH, Halter SA, Schlom J. Expression of the 21,000 molecular weight ras protein in a spectrum of benign and malignant human mammary tissues. Cancer Res 1986;46(5):2511-9.

36. Thor A, Ohuchi N, Hand PH, Callahan R, Weeks MO, Theillet C, Lidereau R, Escot C, Page DL, Vilasi V, et al. Ras gene alterations and enhanced levels of ras p21 expression in a spectrum of benign and malignant human mammary tissues. Lab Invest 1986;55(6):603-15.

37. Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR. Protein kinase c alpha activates raf-1 by direct phosphorylation. Nature 1993;364(6434):249-52.

38. Wu J, Dent P, Jelinek T, Wolfman A, Weber MJ, Sturgill TW. Inhibition of the egf-activated map kinase signaling pathway by adenosine 3',5'-monophosphate [see comments]. Science 1993;262(5136):1065-9.

39. Vaillancourt RR, Gardner AM, Johnson GL. B-raf-dependent regulation of the mek-1/mitogen-activated protein kinase pathway in pc12 cells and regulation by cyclic amp. Mol Cell Biol 1994;14(10):6522-30.

40. Heerdt BG, Houston MA, Augenlicht LH. Potentiation by specific short-chain fatty acids of differentiation and apoptosis in human colonic carcinoma cell lines. Cancer Res 1994;54(12):3288-93.

41. Bracey TS, Miller JC, Preece A, Paraskeva C. Γ-Radiation-induced apoptosis in human colorectal adenoma and carcinoma cell lines can occur in the absence of wild type p53. Oncogene 1995;10:2391-6.

42. Strange R, Li F, Saurer S, Burkhardt A, Friis RR. Apoptotic cell death and tissue remodelling during mouse mammary gland involution. Development 1992;115(1):49-58.

43. Kyprianou N, English HF, Davidson NE, Isaacs JT. Programmed cell death during regression of the mcf-7 human breast cancer following estrogen ablation. Cancer Res 1991;51(1):162-6.

44. Warri AM, Huovinen RL, Laine AM, Martikainen PM, Harkonen PL. Apoptosis in toremifene-induced growth inhibition of human breast cancer cells in vivo and in vitro. J Natl Cancer Inst 1993;85(17):1412-8.

45. Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kilobase fragments prior to or in the absence of internucleosomal fragmentation. EMBO J 1993;12:3679-84.

46. Wilson JW, Wakeling AE, Morris ID, Hickman JA, Dive C. MCF-7 human mammary adenocarcinoma cell death *in vitro* in response to hormone-withdrawal and DNA damage. Int J Cancer 1995;61:502-8.

47. Sumantran VN, Ealovega MW, Nunez G, Clarke MF, Wicha MS. Overexpression of Bcl-X_S sensitizes MCF-7 cells to chemotherapy-induced apoptosis. Cancer Res 1995;55:2507-10.

48. Wang TTY, Phamg JM. Effects of estrogen on apoptotic pathways in human breast cancer cell

line MCF-7. Cancer Res 1995;55:2487-9.

49. Filmus J, Pollak MN, Cailleau R, Buick RN. MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. Biochem Biophys Res Commun 1985;128:898-905.

50. Armstrong DK, Kaufmann SH, Ottaviano YL, Furuya Y, Buckley JA, Isaacs JT, Davidson NE. Epidermal growth factor-mediated apoptosis of mda-mb-468 human breast cancer cells. Cancer Res 1994;54(20):5280-3.

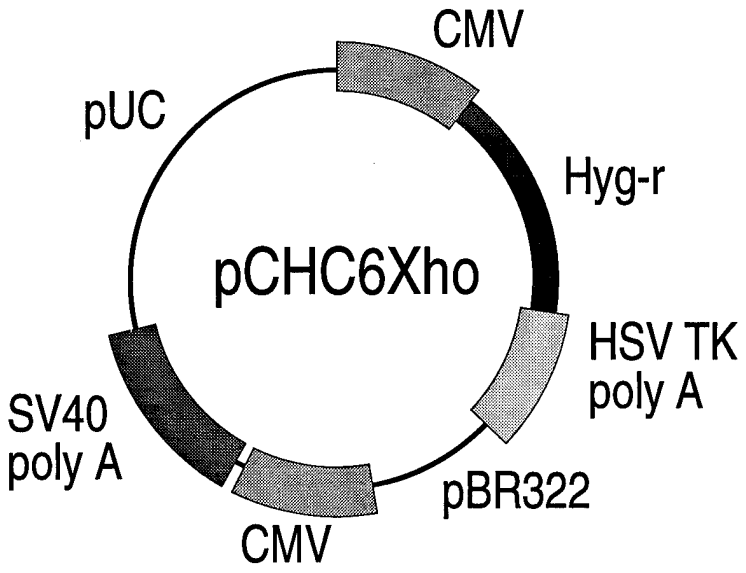
Table I. *In vitro* Kinase Assay of Raf Clones

Cell line	Specific cpm[1]
HCo pool	70,801
Raf 4	1,922,028
Raf 8	1,313,208
Raf 14	425,828
Raf 27	280,228
Raf 35	1,621,068

Raf protein was immunoprecipitated from 100 μg cellular lysate and reacted with γ-^{32}P and a peptide substrate specific for Raf. Reaction mixes were run through phosphocellulose filters to retain phosphorylated peptide, and the filters were counted.

1. Specific cpm values were calculated by subtracting the non-specific counts obtained with no lysate from the total counts.

Figure 1


CMV
pUC
Hyg-r
pCHC6Xho
HSV TK
poly A
SV40
poly A
pBR322
CMV

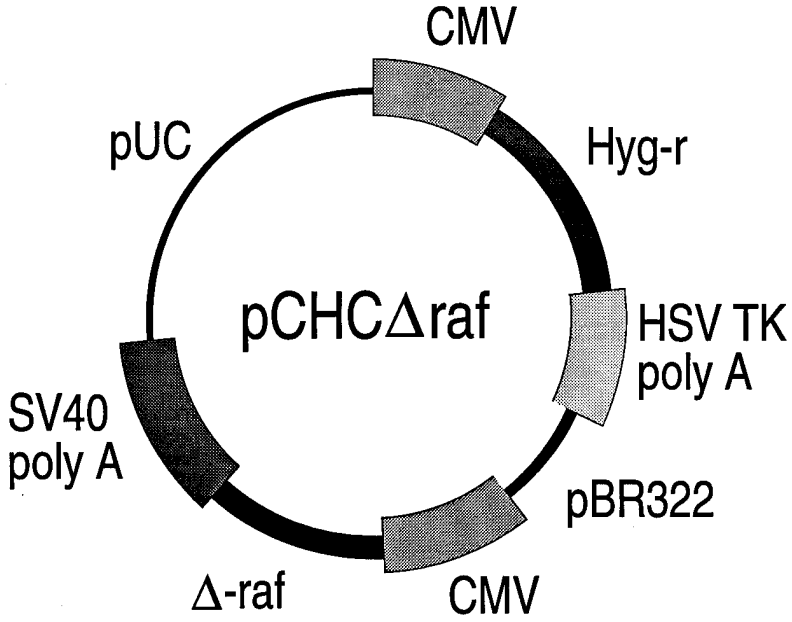

CMV
pUC
Hyg-r
pCHCΔraf
HSV TK
poly A
SV40
poly A
pBR322
Δ-raf
CMV

Figure 2

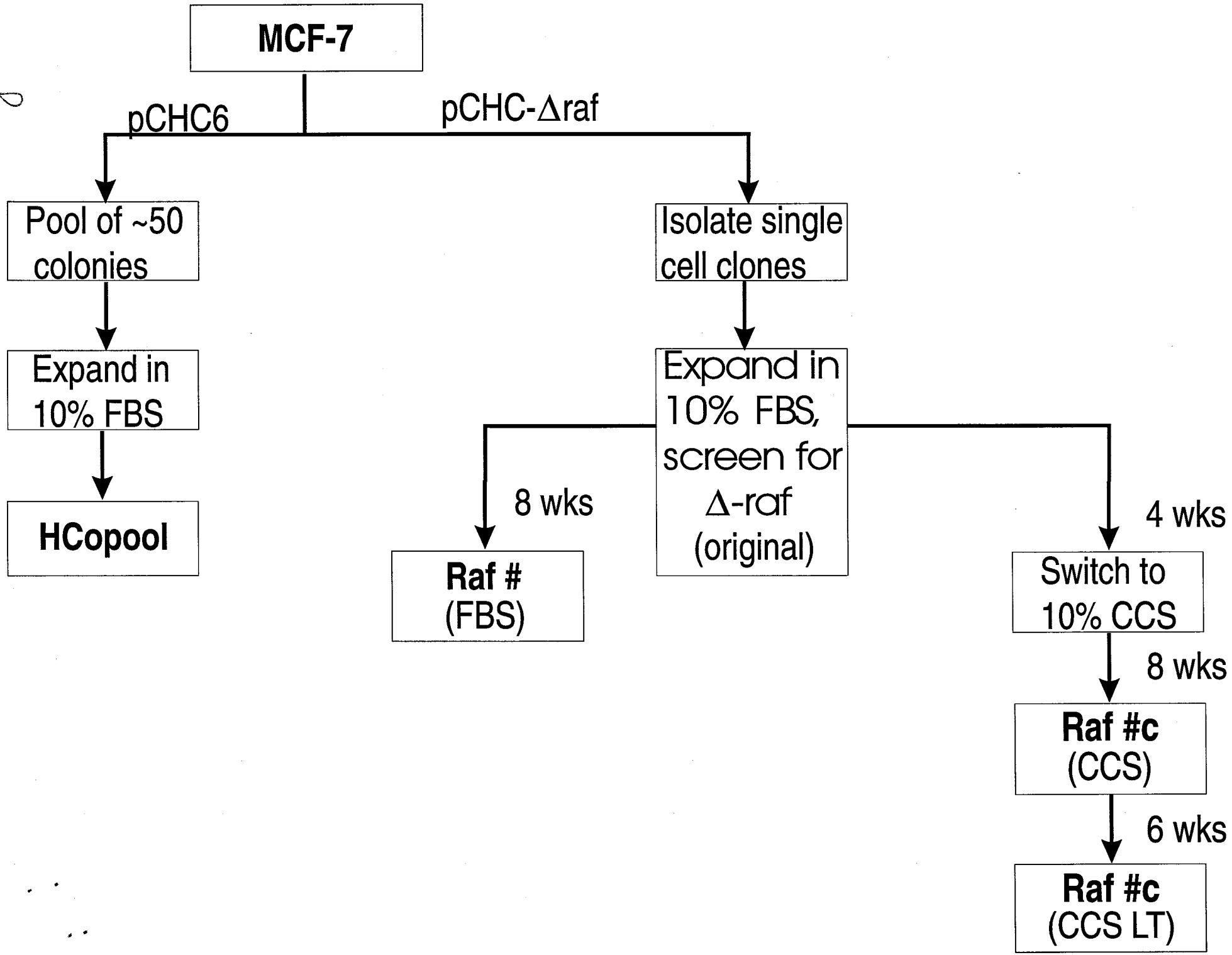

MCF-7
pCHC6
pCHC-Δraf
Pool of ~50 colonies
Expand in 10% FBS
HCopool
Isolate single cell clones
Expand in 10% FBS, screen for Δ-raf (original)
8 wks
Raf # (FBS)
4 wks
Switch to 10% CCS
8 wks
Raf #c (CCS)
6 wks
Raf #c (CCS LT)

Figure 3A

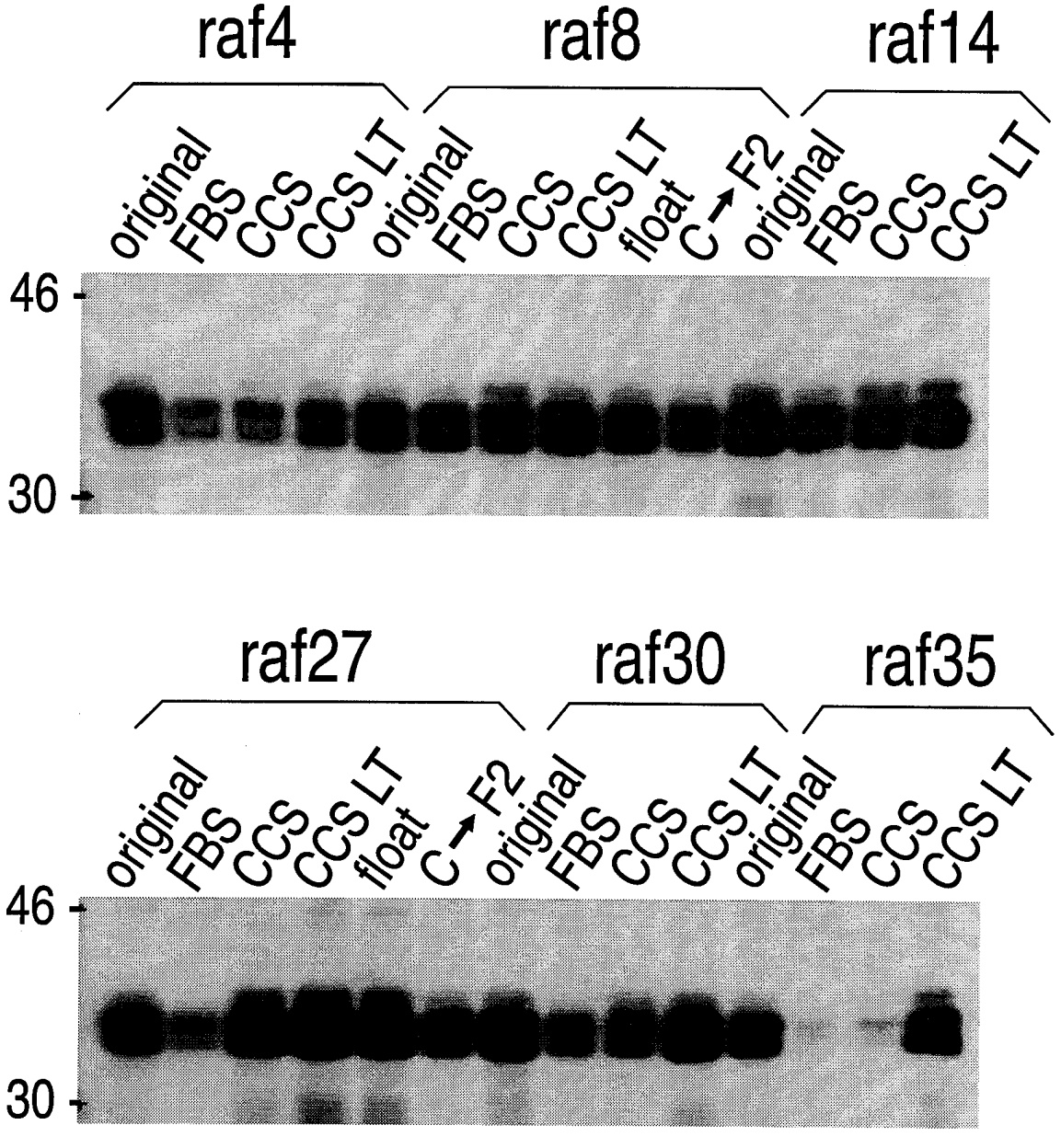

raf4
raf8
raf14
original
FBS
CCS
CCS LT
original
FBS
CCS
CCS LT
float
C → F2
original
FBS
CCS
CCS LT
46
30
raf27
raf30
raf35
original
FBS
CCS
CCS LT
float
C → F2
original
FBS
CCS
CCS LT
original
FBS
CCS
CCS LT
46
30

Figure 3B

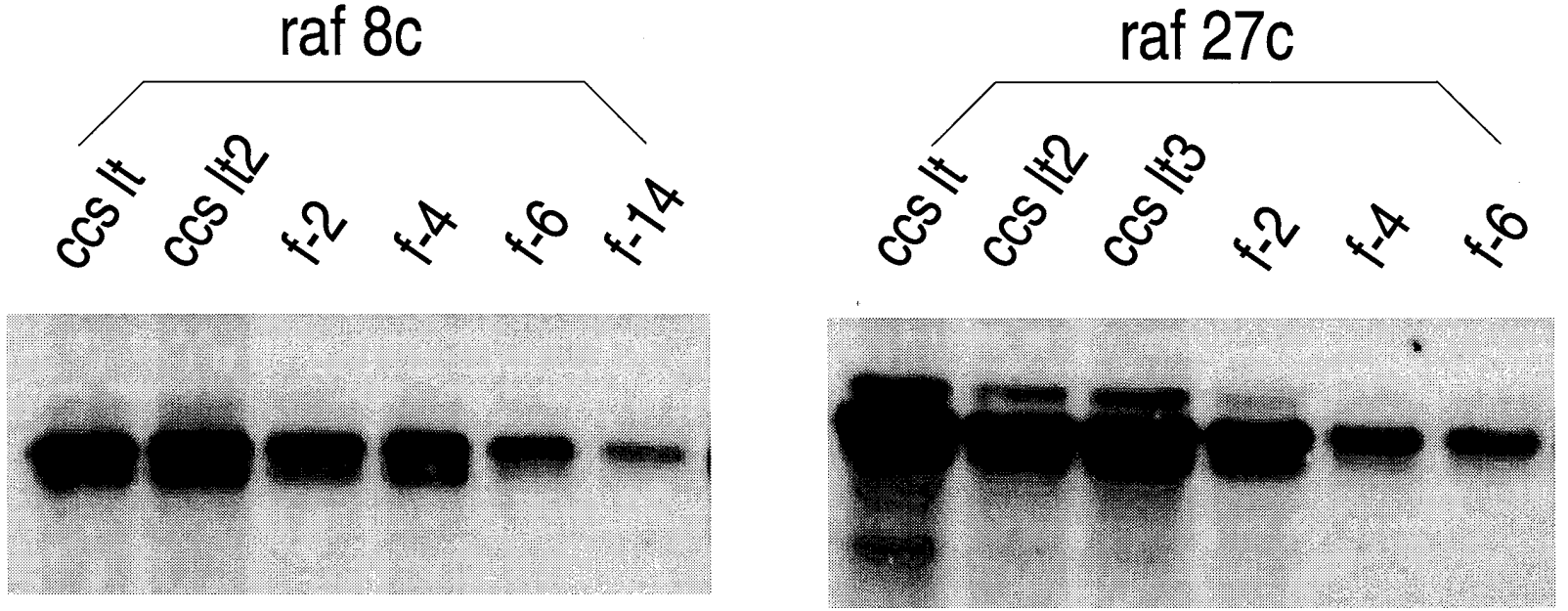

raf 8c
ccs lt
ccs lt2
f-2
f-4
f-6
f-14
raf 27c
ccs lt
ccs lt2
ccs lt3
f-2
f-4
f-6

Figure 4

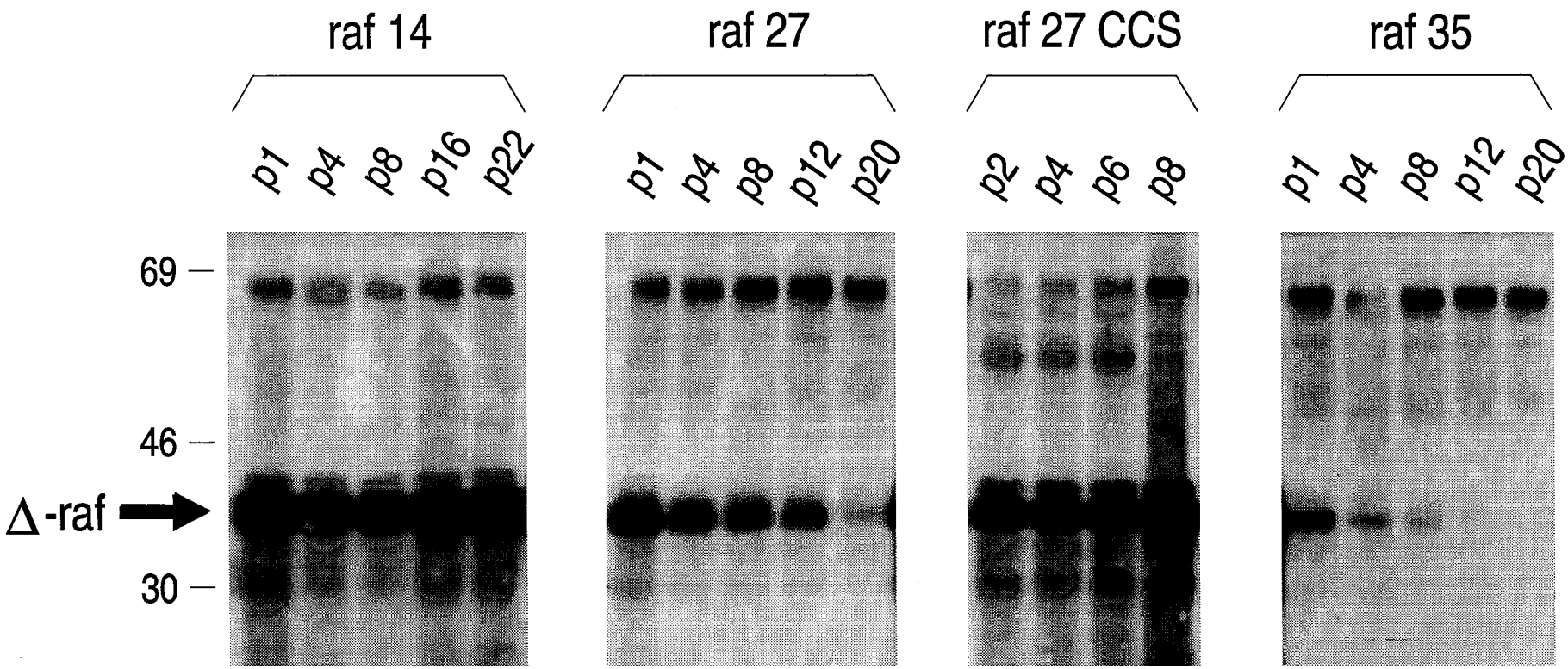

raf 14
raf 27
raf 27 CCS
raf 35
p1
p4
p8
p16
p22
p1
p4
p8
p12
p20
p2
p4
p6
p8
p1
p4
p8
p12
p20
69
46
Δ-raf
30

Figure 5

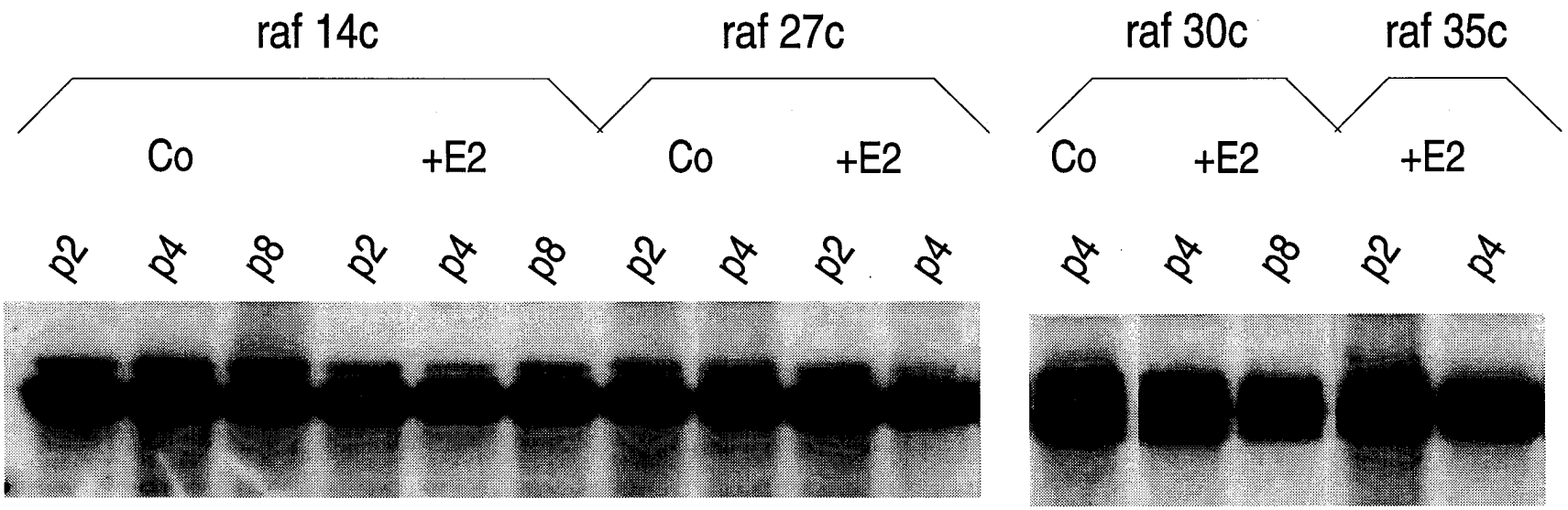

raf 14c
raf 27c
raf 30c
raf 35c
Co
+E2
Co
+E2
Co
+E2
+E2
p2
p4
p8
p2
p4
p8
p2
p4
p2
p4
p4
p4
p8
p2
p4

Figure 6

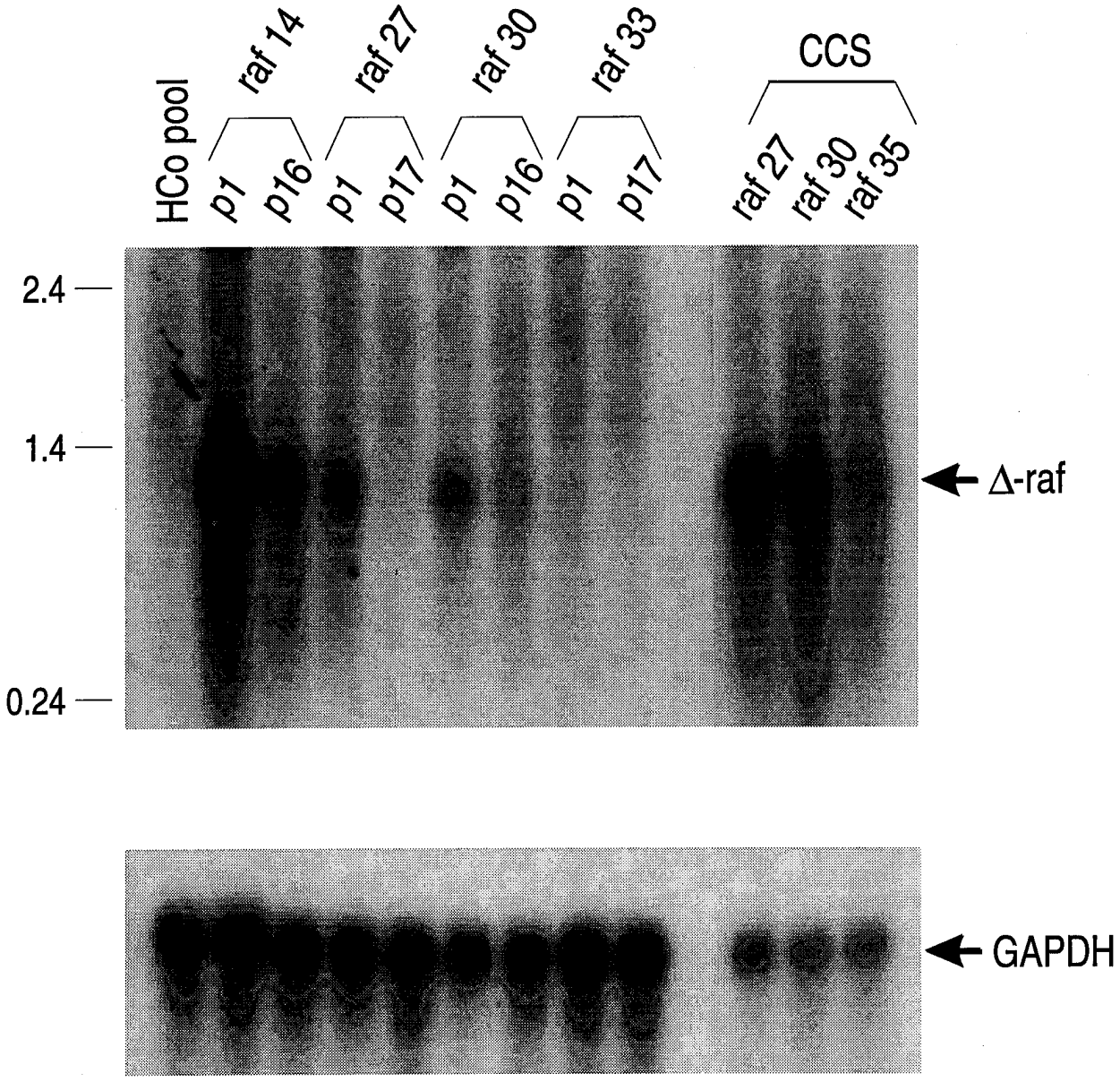

HCo pool
raf 14
p1
p16
raf 27
p1
p17
raf 30
p1
p16
raf 33
p1
p17
CCS
raf 27
raf 30
raf 35
2.4
1.4
0.24
Δ-raf
GAPDH

Figure 1A

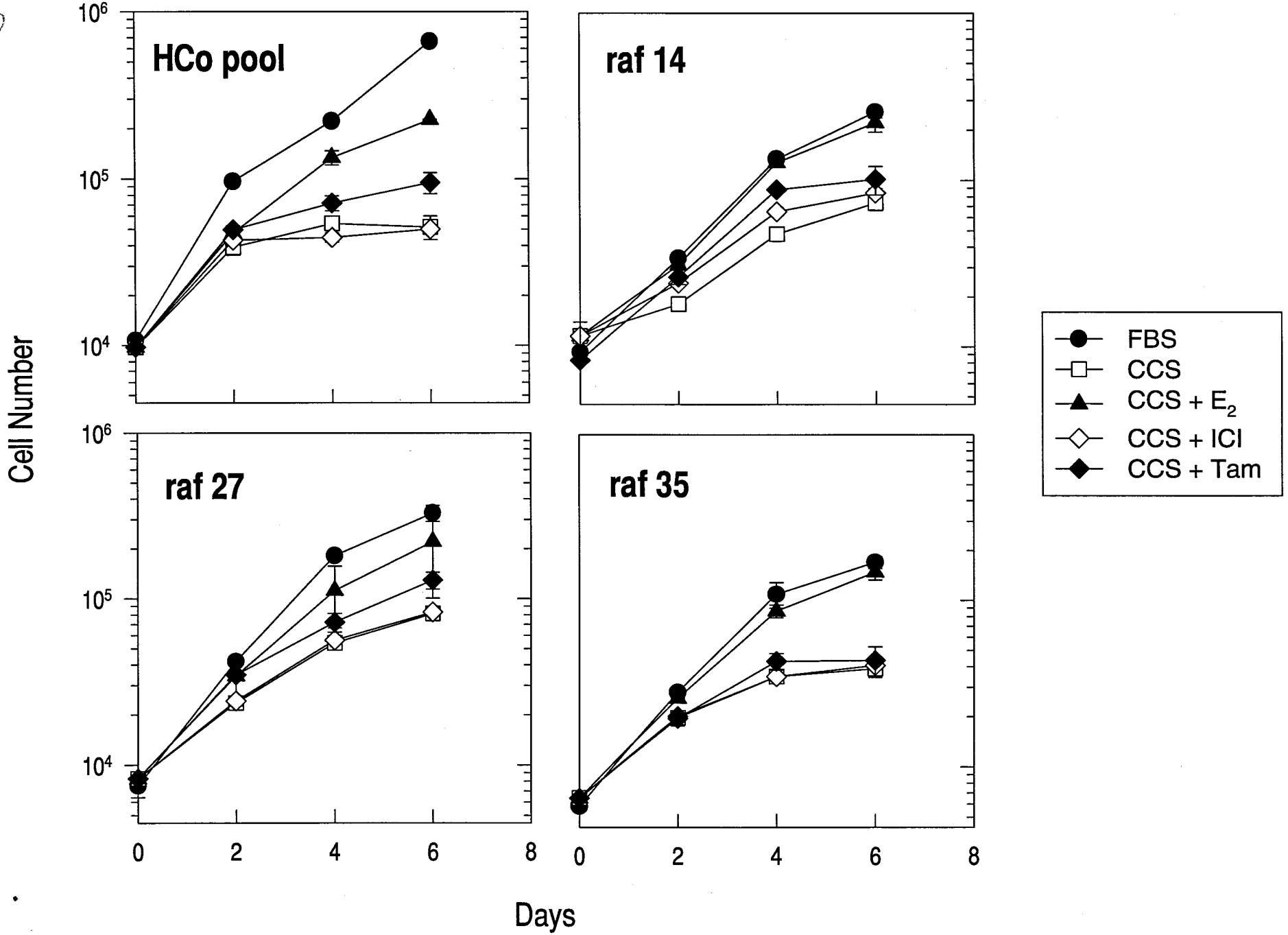
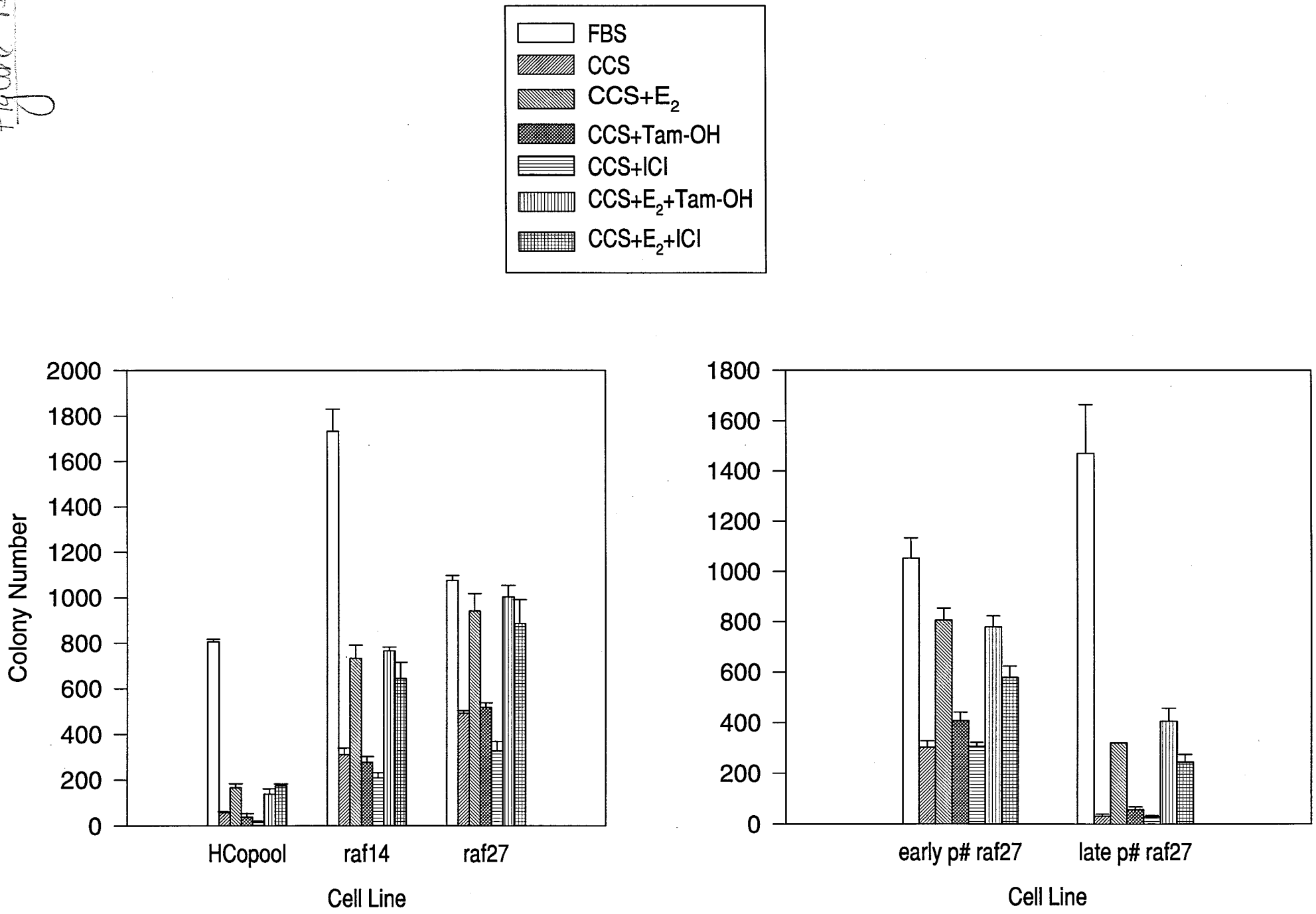
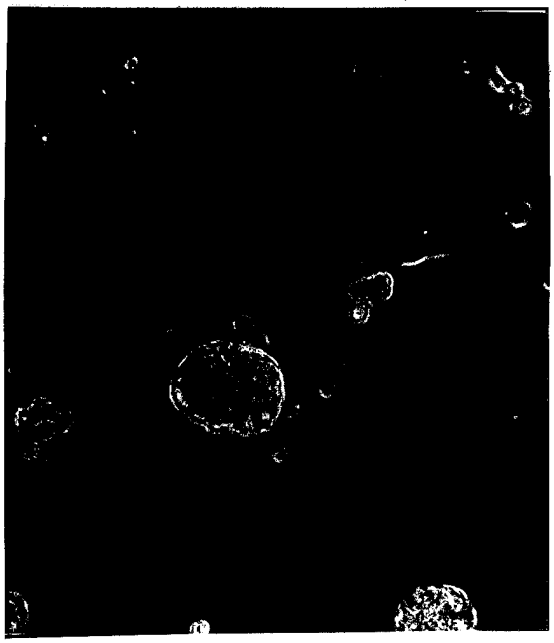

HCo pool
raf 14
raf 27
raf 35
Cell Number
Days
10^6
10^5
10^4
0
2
4
6
8
FBS
CCS
CCS + E_2
CCS + ICI
CCS + Tam

Figure 1B


FBS
CCS
CCS+E2
CCS+Tam-OH
CCS+ICI
CCS+E2+Tam-OH
CCS+E2+ICI
Colony Number
2000
1800
1600
1400
1200
1000
800
600
400
200
0
HCopool
raf14
raf27
Cell Line
1800
1600
1400
1200
1000
800
600
400
200
0
early p# raf27
late p# raf27
Cell Line

Figures 8A+8B



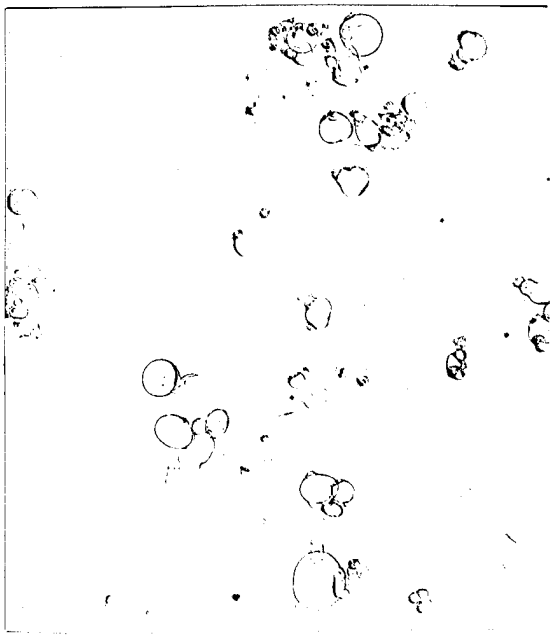
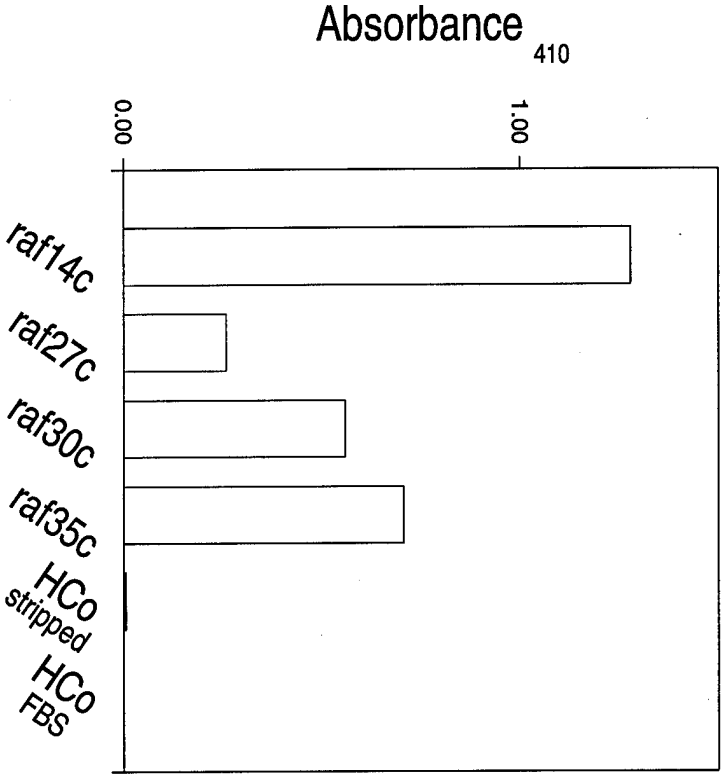


Figure 8C


Absorbance 410
0.00
1.00
raf14c
raf27c
raf30c
raf35c
HCo stripped
HCo FBS

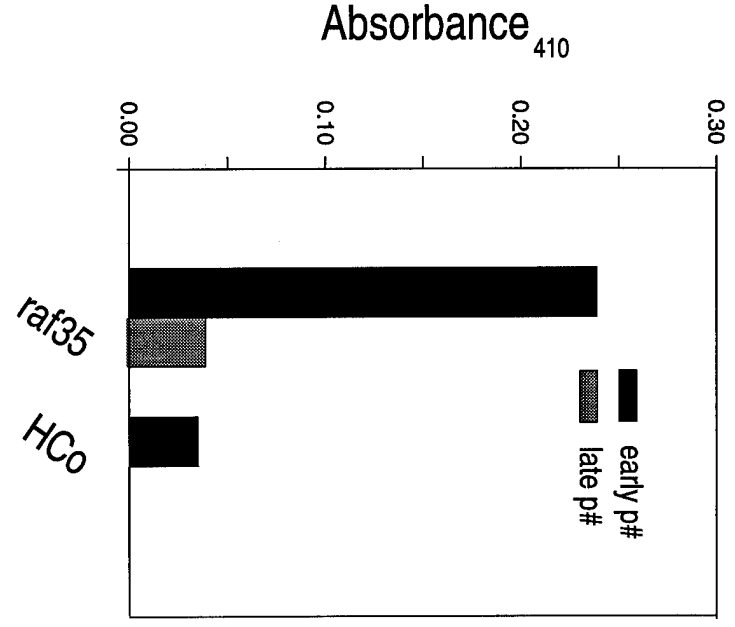
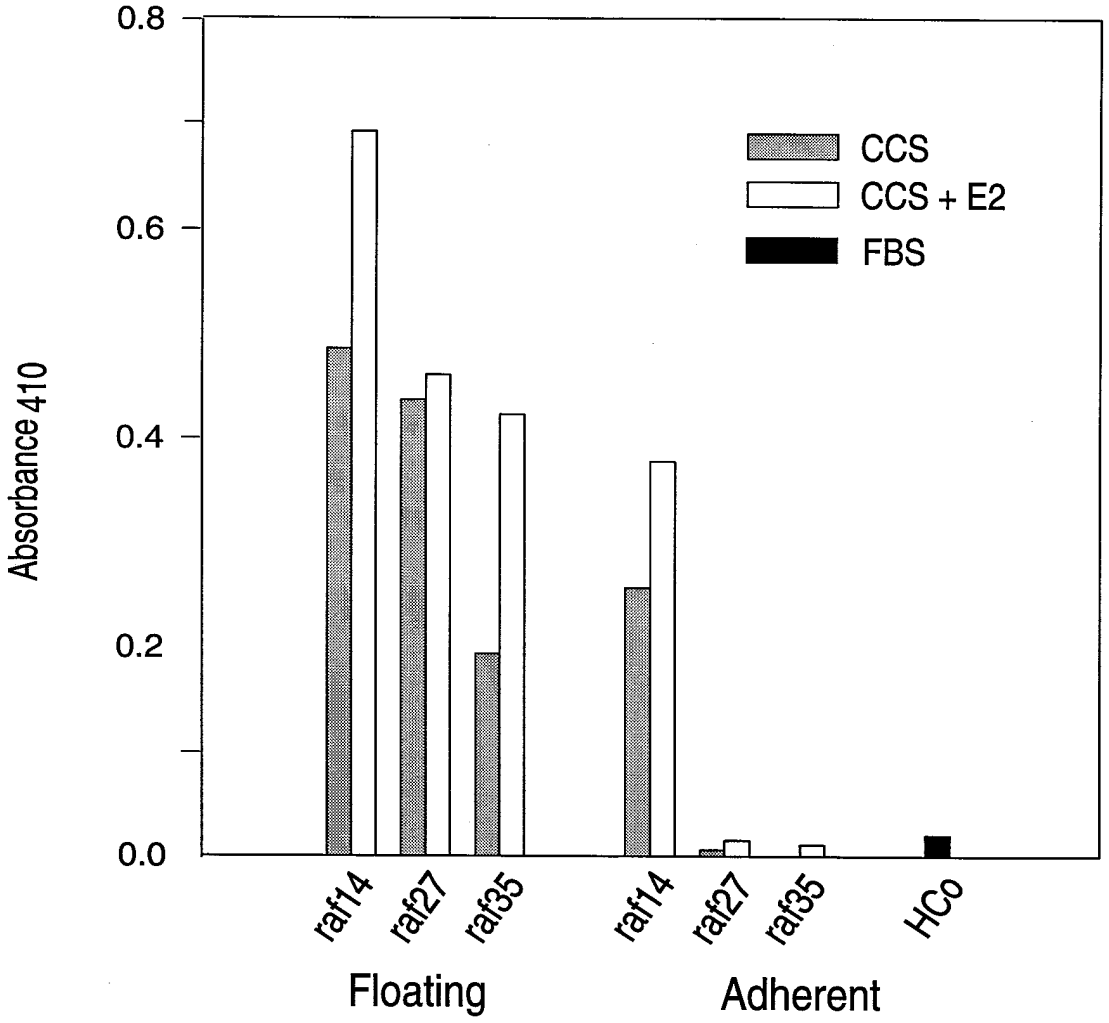

Absorbance 410
0.00
0.10
0.20
0.30
raf35
HCo
early p#
late p#

Figure 8D


0.8
0.6
0.4
0.2
0.0
Absorbance 410
CCS
CCS + E2
FBS
raf14
raf27
raf35
raf14
raf27
raf35
HCo
Floating
Adherent